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Evaluation of Methods for Predicting the Toxicity of Polycyclic Aromatic Hydrocarbon Mixtures

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Risk assessments of polycyclic aromatic hydrocarbon mixtures are hindered by a lack of reliable information on the potency of both mixtures and their individual components. This paper examines methods for approximating the toxicity of polycyclic aromatic hydrocarbon (PAH) mixtures. PAHs were isolated from a coal tar and then separated by ring number using HPLC. Five fractions (A−E) were generated, each possessing a unique composition and expected potency. The toxicity of each fraction was measured in the *Salmonella*/mutagenicity assay and the Chick Embryo Screening Test (CHEST). Their abilities to induce ethoxyresorufin-*O*-deethylase and to inhibit gap junction intercellular communication in rat liver Clone 9 cells were also measured. In the *Salmonella*/mutagenicity assay, fractions were predicted to have potencies in the order C > D > E > B > A. Toxic equivalency factors (TEFs) for fractions A−E were in the order E ≥ D > C > B > A. TEF values were 20 652, 20 929, 441, 306, and 74.1 μg of BaP equiv/g, respectively. A lack of agreement between assay-predicted potencies and chemical analysis-predicted potencies was observed with other assays and other methods of calculation. The results demonstrate the limitations of using a single method to predict the toxicity of a complex PAH mixture.

Introduction

Chemical mixtures complicate toxicity assessments because of their often complex and variable composition. To produce consistent and reliable estimates of toxicity for these mixtures, standardized approaches have been developed. These methods include (i) using toxicity data derived from experiments with the mixture of interest, (ii) extrapolating toxicity data from a similar mixture, or (iii) summing the toxicities of individual components (*1*). Data derived directly from the mixture of interest is the most preferable. In the case of PAH mixtures, however, mixture-specific toxicity data are often not available, particularly with respect to carcinogenicity. Component-oriented approaches are the most common technique for calculating risks from these materials. Unfortunately, there is a lack of data for many polycyclic aromatic hydrocarbons (PAHs), necessitating the use of approximations.

Toxic equivalency factors (TEFs) were first developed for halogenated aromatic compounds such as polychlorinated biphenyls (PCBs) as a means of ranking their toxicity relative to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (*2*). Several limitations apply to the use of TEFs for TCDD-like effects, and important assumptions must be met. Most importantly, the toxic action must be mediated by the aromatic hydrocarbon (Ah) receptor. Compounds also must bioaccumulate and have a multi-ringed and chlorinated aromatic structure. Finally, response additivity and similar dose−response curves are implicit assumptions for TCDD-like and all other TEFs (*3*, *4*).

Several limitations of this approach have been identified. Resistant strains of experimental animals have complicated developing consistent TEF values for a range of species (*5*, *6*). Nonadditive responses have been observed to occur between individual PCB congeners, which severely impairs the utility of TEFs (*7*). Finally, naturally occurring compounds that bind with the Ah receptor can interfere with or even overwhelm responses caused by chlorinated aromatics (*8*−*10*). When these factors are controlled for, TEFs have been demonstrated to accurately predict the toxicity of chlorinated aromatic hydrocarbons in complex mixtures (*11*, *12*).

Similar approaches with TEFs for PAHs have proved more difficult. In contrast to TEFs for halogenated compounds, which are based on Ah receptor affinity, PAH TEFs are intended for all cancer effects and thus encompass a wider range of endpoints. Chu and Chen and Clement Associates were the first to develop TEFs for PAHs (*13*). Their work was eventually synthesized and adapted by Nisbet and LaGoy (*13*) into a set of commonly referenced order of magnitude estimates. In 1993, the U.S. EPA's Office of Environmental Health Assessment (OHEA) issued a provisional guidance document on the state of knowledge for implementing TEFs for PAHs (*4*). This document emphasized that a lack of knowledge about PAH toxicity, minimal information about PAH interactions, and minimal information about promotional effects limit development of TEFs for this class of compounds. It was only possible then to calculate "estimated orders of potential potency" for those identified as class B2 carcinogens. Estimated orders of potential potency are referred to here as potential potencies (PPs). Values in OHEA's summary, as in Nisbet and LaGoy's, were order of magnitude values. Prior to this, the U.S. EPA separated PAHs into carcinogens and noncarcinogens, applying the Cancer Slope Factor (CSF) for BaP to all carcinogenic PAHs (*13*). The U.S. EPA's Proposed Guidelines for Carcinogen Risk Assessment state that at the current time not enough information exists to use PAH TEFs for risk assessments. Only PPs may be used. The 1996 proposed guidelines will replace the current 1986 guidelines when adopted (*14*).

Many studies have examined how the composition of complex PAH affects observed toxicity. Some have shown that toxicity may not necessarily be predicted by BaP content alone. Weyand et al. dosed B6C3F1 mice with coal tar in their diets or an amount of BaP equal to that in the ingested coal tar (*15*). They found significant differences in sites of tumors and numbers of tumors between BaP and coal tar groups. Matsumoto et al. (*16*) surveyed PAH levels on air particulate matter and measured mutagenicity of these particulates with the *Salmonella*/microsome assay. Their results showed no correlation between mutagenicity and BaP

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content in air samples. Chuang et al. (*17*) fractionated an organic extract of air samples. They found that most of the mutagenicity, as measured by the *Salmonella*/microsome assay, was produced by a fraction enriched in three- and four-ring alkylated PAHs. These results indicate that a risk assessment based on the assumption that all PAHs can be directly compared to BaP may be inaccurate.

The objectives for this research were (i) to examine the contribution PAH fractions (based on different ring numbers) make toward overall toxicity of a complex mixture and (ii) evaluate the applicability of toxic equivalency factors (TEFs) and other approximations for these compounds using a series of rapid in vitro and in vivo bioassays. To accomplish this, PAHs were isolated from a sample of coal tar and then separated via normal-phase HPLC into five fractions. Each fraction was tested in the *Salmonella*/microsome assay, the Chick Embryo Screening Test (CHEST), and the Gap Junction Intercellular Communication (GJIC) assay and their ability to induce cytochrome P450 enzymes in hepatic cells.

Cancer is believed to be a multi-stage process including an initiating event and a series of events where the growth of the initiated cell is promoted and the cell eventually progresses to be a rapidly growing malignant cell. While the initiation of cancer often results from a genotoxic event, promotion and progression may include a variety of epigenetic events (*18*). In the present study, bioassays were chosen for their ability to measure both initiation-related and promotion-related effects of carcinogens. Mutations of DNA, such as those measured by the *Salmonella*/microsome assay, are known to be important initiating events (*19*, *20*). Induction of enzymes of the cytochrome P450 family (e.g., EROD) is an essential step in the activation of carcinogens such as PAHs prior to initiation. In terms of promotion, organ damage can lead to subsequent proliferation and serve to selectively promote the growth of initiated cells (*21*). Farber and Tsuda (*22*) described the use of partial hepatectomy followed by a dose of carbon tetrachloride as a means of inducing proliferation in order to promote tumors following a dose of a suspected initiator. The CHEST assay is employed here as a means to measure the potential of PAH mixtures to produce liver damage that could lead to subsequent cell proliferation. Inhibiting cell-to-cell communication is also believed to promote tumor growth by eliminating signals that instruct an initiated cell to stop dividing (*18*, *21*, *23*). Measurements of gap junction communication serve as an indicator of PAHs to impair this function. By failing to take both initiation and promotion effects into account, TEFs and PPs may be limited in their ability to predict the activity of PAH mixtures. It is believed that, by examining both types of end points, a clearer understanding of the relationship between composition and toxicity can be obtained.

Materials and Methods

Methylene chloride, chloroform, hexane, and heptane (all HPLC grade) were purchased from VWR Scientific (Suwanee, GA). PAH standards for chemical analysis were purchased from Supelco (Bellefonte, PA). Alumina, 80−200 mesh, Brockman Activity I, was purchased from Fisher Scientific (Pittsburgh, PA). Coal tar (CT) was collected in precleaned glass jars from a closed manufactured gas plant facility in the central United States and stored at 5 ± 2 °C.

PAHs were first isolated from CT with methods modified from those of Schiller and Mathiasson (*24*). Approximately 4.5 g of CT was dissolved in 20 mL of chloroform. To this, 43.5 g of alumina was added, and the mixture was dried under vacuum until it flowed freely. This material (8 g) was added to the top of a 2.2 cm × 60 cm glass chromatography column containing 24 g of alumina. The column was fitted with a stopcock and ceramic frit. PAHs were isolated in two fractions. The first was generated by adding 80 mL of hexane and collecting 60 mL. The second was generated by adding 200 mL of toluene and collecting 200 mL. Flow was kept to approximately 3 mL/min. This produced fraction 1 containing alkanes and 2−3-ring PAHs and fraction 2 containing 3−5+-ring PAHs. This was repeated until all of the CT/alumina mixture was consumed.

HPLC fractions were generated by using a preparative-scale version of the methods of Wise et al. (*25*). A 22 mm × 300 mm μBondapak aminopropylsilane column, 10 μm particle size, 125 Å pore size (Waters Associates, Milford, MA) was used for the separation. The HPLC was comprised of a Waters 600E multisolvent delivery system coupled to a Waters 996 photodiode array detector. Detection was set at 254 nm. The mobile phase was 3:1 hexane:heptane at 7 mL/min for 67 min, ramping to 9 mL/min at 68 min. During the increase in flow, the mobile phase changed to 95% 3:1 hexane:heptane and 5% methylene chloride. The total run time was 95 min to allow polar compounds to be removed before the next run. Fractions were collected at 45-s intervals by a Waters fraction collector and recombined based on retention times of standards.

Standards were then used to select fraction cut points on the HPLC. The standards included the individual PAH compounds and a 13-component PAH priority pollutant mixture from Supelco (Bellefonte, PA). Alumina fraction 1 was separated into HPLC fractions A1, B1, and C1. Fraction A1 was collected from the HPLC column between 10.2 and 20.9 min. Fraction B1 was collected between 20.9 and 26.6 min. Fraction C1 was collected between 26.6 and 40.5 min. Alumina fraction 2 was separated into HPLC fractions A2−E. Fraction A2 was collected between 11.8 and 13.4 min, fraction B2 was collected between 20 and 37.2 min, and fraction C2 was collected between 37.2 and 47.5 min. Fraction D was collected between 47.5 and 56.3 min, and fraction E was collected between 56.3 and 74.9 min. Fractions A1 and A2, B1 and B2, and C1 and C2 were combined by weight in proportion to the weights of the two alumina column fractions. Table 1 presents analytical data for each of the fractions. Fractions were analyzed with an HP-5890 series II GC coupled to an HP 5972-MS. The column was a 30 m, 0.25 mm i.d. HP5 MS. The temperature program began at 35 °C for 6 min, ramped 5 °C/min to 300 °C, and held for 30 min. All analyses were completed using the scan mode from 50 to 550 m/z. Calibration solutions were prepared for PAHs at five concentrations ranging from 0.02 to 1 μg/mL by diluting a commercially available solution containing the analytes of interest (typically NIST SRM 2260). At a minimum, 10% of the samples analyzed on the GC−MS were standards.

Salmonella typhimurium tester strain TA98 was kindly provided by B. N. Ames (University of California, Berkeley). Fractions were resuspended in dimethyl sulfoxide (DMSO) and tested on duplicate plates in two independent experiments at five dose levels (1, 2, 5, 10, and 20 mg/mL). Fractions were tested only in the presence of metabolic activation (S9 mixture prepared with S9 fraction of Aroclor 1254-induced Sprague−Dawley rat liver). Media preparation and other methods followed those of Maron and Ames (*26*). All bioassays included positive and solvent controls. Data were analyzed using GeneTox software kindly provided by L. D. Claxton (U.S. EPA, Research Triangle Park, NC). Data were fit to the Stead model to generate values for mutagenicity and toxicity as described by Stead et al. (*27*).

Rat liver Clone 9 cells (ATCC, CRL 1439, passage 17) were used for GJIC and Ethoxyresorufin-*O*-deethylase (EROD) assays. Cultures were used within 10 passages after being received and maintained in Ham's nutrient mixture F-12 containing 10% fetal bovine serum. For EROD measurements, cells were seeded at 10 000 cells/cm^2 and incubated until they became approximately 70% confluent. For the GJIC

TABLE 1. PAHs (μg/g), Benzo[*a*]pyrene (BaP) Equivalents (μg/g), and Percent Unknown by Weight in Fractions A–E[a]

	fraction A	fraction B	fraction C	fraction D	fraction E
naphthalene	21354	65.9	73.8	43.8	54.6
alkyl naphthalenes	89375	404	689	284	508
acenaphthylene	10395	1049	1550	691	1500
acenaphthene	3063	68.4	174	74.6	78.1
fluorene	19642	2865	296	102	164
alkyl fluorenes	17907	12472	957	189	1373
phenanthrene	486	101937	2458	286	240
anthracene	209	18407	351	64.3	1000
alkyl PHE/ANT	780	145385	12685	206	868
dibenzothiophene	1127	6103	72.2	23.3	17.5
alkyl DBT	6315	6905	331	60.1	77.6
fluoranthene	94.2	1664	38196	51.4	50.5
pyrene	100	145	42784	64.6	54.4
alkyl FNT/PYR	31.4	257	51027	49.7	231
benz[*a*]anthracene	14.2	15.9	36027	328	31.1
chrysene	15.1	19.0	32883	713	15.2
alkyl chrysenes	25.6	385	60406	7091	2178
benzo[*b*]fluoranthene	17.1	7.3	65.5	15960	8730
benzo[*k*]fluoranthene	7.7	4.3	100	6020	1140
benzo[*e*]pyrene	5.3	3.4	97.9	11480	285
benzo[*a*]pyrene	4.4	3.6	27.3	17260	70.4
perylene	3.5	2.1	61.8	1660	2810
indeno[1,2,3-*cd*]pyrene	5.7	7.9	38.4	175	9330
dibenz[*a*,*h*]anthracene	1.5	1.2	68.6	226	3770
benzo[*ghi*]perylene	8.1	2.0	3.1	439	7360
total PAHs	177 106	298 199	281 448	63 554	41 951
total carcinogenic PAHs[b]	65.8	59.2	69210	40682	23087
Nisbet and LaGoy TEFs[c]	74.1	306	4411	20652	20929
U.S. EPA PPs[d]	9.9	8.2	4039	19200	5661
% unknown	82.3	70.2	71.9	93.6	95.8

[a] PHE, phenanthrene; ANT, anthracene; DBT, dibenzothiophene; FNT, fluoranthene; PYR, pyrene. [b] Total carcinogenic PAHs is the sum of all class B2 carcinogens. [c] TEFs, toxic equivalency factors from Nisbet and LaGoy (*13*). [d] PPs, potential potencies from U.S. EPA (*4*).

assay, cells were seeded at 50 000 cells/cm^2 and incubated for 24 h before use.

EROD assays were performed in triplicate with Clone 9 cells using the 96-well microplate fluorometric assay described by Kennedy and Jones (*28*) and by Kennedy et al. (*29*). Treatments were added wells to give a final DMSO concentration of 0.5%. Fractions were dissolved in DMSO to give concentrations of 5, 2, 1, 0.5, and 0.1 mg/mL. Resorufin concentrations were measured using a microplate fluorescence reader (Bio-Tek FL600, Bio-Tek Instruments, Inc., Winooski, VT). Total protein was determined using a BCA-200 Protein Assay Kit from Pierce (Rockford, IL). Bovine serum albumin (BSA) was employed as a protein standard. Excitation and emission wavelengths were set to 530/25 and 590/35 nm, respectively, for the EROD assay. Absorbance was measured at 562 nm for protein quantitation. Data were analyzed using a one-way ANOVA followed by Tukey's test. Significance was set at $p < 0.05$.

Fertilized white leghorn hen's eggs form the same flock were obtained from the Texas A&M Poultry Science Center (College Station, TX). Eggs were left at room temperature (22 $\pm$ 2 °C) for 24 h and then were incubated at 37.5 °C and 80% relative humidity in an automatic incubator. Eggs were turned every 3 h. After 4 d of incubation, all eggs were candled. Those that were infertile or contained dead embryos were discarded. Remaining eggs were weighed and cleaned at the blunt end with 70% ethanol. A small dental drill was used to make an injection opening into the air sac. Fractions dissolved in corn oil plus 5% DMSO were injected aseptically into the yolk at 1 mL of sample/kg of egg weight. Concentrations were 2, 1, 0.5, 0.25, and 0.125 mg/mL. A solvent control was also included. Following injection, the hole was sealed with paraffin, and the eggs were returned to the incubator. Embryos were monitored every other day for 18 d for signs of mortality. Dead embryos were discarded. After 18 d, all remaining embryos were dissected for signs of liver necrosis and green liver. Data were analyzed using an R $\times$ C χ^2 with $p < 0.05$ followed by Fisher's exact test with $p < 0.05$.

GJIC was measured in Clone 9 cells by dye coupling. The rate constant of dye transfer between cells was measured in triplicate using the fluorescence recovery after photobleaching (FRAP) technique used by Barhoumi et al. (*30*). Cells were dosed with 1 μL/mL medium of each fraction at five doses (20, 10, 5, 2, and 1 mg/mL) in DMSO for 24 h. Following incubation at 37.5 °C, the medium was removed and replaced with serum free medium. Ten microliters of a 5-carboxyfluoresceindiacetate (CFDA) solution was added, and the cells were incubated again at 37.5 °C for 15 min. After dye was loaded, the medium was replaced, and cells were photobleached and scanned for fluorescence recovery with a Meridian Ultima confocal workstation (Meridian Instruments, Okemos, MI). Results from each treatment were compared using a one-way ANOVA and Tukey's test. Significance was set at $p < 0.05$.

Results and Discussion

Fractionation. Column chromatography followed by normal-phase HPLC separation proved successful at both separating PAHs from a parent mixture and isolating PAHs based on ring number. Concentrations of individual compounds along with calculations of TEFs, potential potencies (PPs), and sums of total carcinogenic PAHs (cPAHs) are presented in Table 1. Using the methods of Nisbet and LaGoy (*13*) gave similar TEFs for fractions D and E with 20 652 and 20 929 μg of BaP equiv/g, respectively. Fraction C had the next highest value with 4411 μg/g. Fractions B and A had the lowest values with 306 and 74.1 μg/g, respectively. U.S. EPA's PPs gave similar

TABLE 2. Total Revertants in the *Salmonella*/Microsome Assay Produced by Each Fraction and DMSO Control

dose (mg/pt)	DMSO	fraction A	fraction B	fraction C	fraction D	fraction E
0.05	46 ± 3	50 ± 6	57 ± 9	111 ± 12	189 ± 10	115 ± 10
0.01	46 ± 3	52 ± 8	62 ± 5	143 ± 17	238 ± 12	142 ± 12
0.25	46 ± 3	55 ± 14	70 ± 4	213 ± 40	266 ± 15	172 ± 16
0.5	46 ± 3	64 ± 10	81 ± 12	298 ± 96	282 ± 32	178 ± 21
1	46 ± 3	65 ± 18	88 ± 12	313 ± 11	244 ± 20	167 ± 12
			Stead Model Results[a]			
adequacy	na	0.57	0.00	3.51	2.48	0.96
p value, adequacy	na	0.751	0.998	0.173	0.289	0.62
toxicity value	na	0.37	0.65	32.01	41.32	20.82
p value, toxicity	na	0.541	0.42	0.000	0.000	0.000
mutagenicity value	na	20.83	73.38	1472.52	1034.79	466.9
p value, mutagenicity	na	0.000	0.000	0.000	0.000	0.000

[a] Values from the Stead model were determined by GeneTox software as described in ref *27*. Fit was judged sufficient if $p > 0.05$. Fractions were judged toxic and mutagenic if $p < 0.05$. na, not applicable.

values although the value for fraction E is reduced to approximately one-third of the Nisbet and LaGoy value. Fraction C had the greatest amount of total cPAHs with 69 210 μg/g. Fraction D had 40 682 μg/g, and fraction E had 23 087 μg/g. Fractions A and B had similar amounts of 65.8 and 59.2 μg/g, respectively. Totals of all measured PAHs were highest in fractions B and C followed by fraction A, then D, and then E.

Chemical analysis indicated that the combined alumina−HPLC separation was able to isolate five distinct PAH fractions. The different composition of each fraction would suggest that appreciable differences in toxicity should be anticipated. The amount of unknown material in each fraction emphasizes the uncertainty that is introduced by choosing to focus on only one class of compounds within a complex mixture. Analysis of alkanes, N-, O-, and S heterocycles as well as polymeric compounds would help to reduce the amount of unknowns, but their activities are more poorly characterized than those of PAHs.

***Salmonella*/Microsome Assay**. Table 2 presents results of the *Salmonella*/microsome assay. Using the 2-fold rule, fractions A and B failed to produce a positive response at any of the doses tested. DMSO alone produced 46 ± 4 revertants/plate while A and B produced maximal responses of 65 ± 18 and 88 ± 12, respectively. Fraction C was weakly positive at 1 and 2 mg/mL. Doses of 5, 10 and 20 mg/mL were strongly positive. Fraction D produced strongly positive responses at all five doses. The minimum response for this fraction was 190 ± 10 revertants/plate at 1 mg/mL. Fraction E produced weakly positive responses at all five doses. The greatest number of revertants/plate produced by this fraction was 178 ± 21 at 10 mg/mL.

GeneTox software was used to fit data from the Ames assay to the Stead model. This approach fits a nonlinear regression of up to four terms to counts of revertant colonies. The curve contains an exponential decay function to account for toxicity. Data presented are the results of likelihood ratio tests as described by Stead et al. (*25*). The first is for the adequacy of fit, and any value corresponding to $p > 0.05$ was considered acceptable. The next is the toxicity value. Any value corresponding to $p < 0.05$ was considered toxic. The final term is the mutagenicity value. As with toxicity, any value corresponding to $p < 0.05$ was considered mutagenic.

All fractions gave an adequacy value that corresponded to $p > 0.05$. For fraction A, the Stead model produced a toxicity value of 0.37 ($p = 0.541$), indicating a lack of toxicity. The mutagenicity value was 20.83 ($p = 0.000$), indicating a positive mutagenic response. Similar results were obtained for fraction B. Fraction C was toxic at the highest doses, producing a value of 32.01 ($p = 0.000$). This fraction also gave the strongest mutagenic response of 1472.52 ($p = 0.000$). Fraction D was also toxic (toxicity value = 41.32, $p = 0.000$) and mutagenic (mutagenicity value = 103.79, $p = 0.000$). Similarly, fraction E was toxic at the highest doses (toxicity value = 20.82, $p = 0.000$) while mutagenic at lower doses (mutagenicity value = 466.9, $p = 0.000$).

Using the Stead model, results of the *Salmonella*/microsome assay predict mutagenicity in the order C > D > E > B > A. The sum of total cPAHs is the only calculation method that produces results similar to this. Both TEFs and PPs predict greater toxicity for fraction D than fraction C. Total PAHs also fail to predict genotoxicity in the orders seen here.

EROD Induction. Results of EROD induction measurements are presented in Figure 1. At the lowest dose of 0.1 mg/mL, fractions A and B induced responses that were not significant from DMSO. This pattern was consistent for all doses of fractions A and B. At a dose of 0.1 mg/mL, fractions C and D induced responses that were similar to each other of 114.24 ± 7.79 and 128.44 ± 4.27 pmol of resorufin liberated min^{-1} (mg of protein)$^{-1}$. Fraction E produced a response of 149.27 ± 9.81 pmol min^{-1} (mg of protein)$^{-1}$ at a dose of 0.1 mg/mL, which was significantly greater than that of all other fractions. At 0.5 mg/mL, fraction E again had the highest response followed by fraction D and then fraction C. Responses of fractions C−E were significant from each other at $p < 0.05$. The same was observed for doses of 1 and 2 mg/mL. At 5 mg/mL, fractions C and D produced similar responses of 478.33 ± 24.2 and 530.01 ± 23.68 pmol min^{-1} (mg of protein)$^{-1}$, respectively. Fraction E again produced a significantly greater response than the other fractions at this dose, 651.25 ± 48.6 pmol min^{-1} (mg of protein)$^{-1}$.

The pattern of EROD induction was similar to that predicted by TEF values. From TEFs, expected toxicities are in the order E ≥ D > C > B > A. EROD induction potency followed the same order. Responses measured with this assay, however, imply a greater difference in potencies between fractions D and E than predicted, while the difference between fractions C and D was smaller than predicted. Despite these discrepancies, TEFs make the best prediction of EROD induction of the three methods examined.

CHEST Assay. Mortality among chick embryos dosed with each of the five fractions was similar to solvent control for fractions A and B. Mortalities in the 2.0 mg/mL groups for fractions C−E were significantly greater than control as was the mortality in the 0.25 mg/mL group for fraction D (Table 3). At 2.0 mg/mL, fractions C−E caused 21, 20, and 19 mortalities out of 25 embryos, respectively. Solvent controls for this dose level exhibited 8 mortalities per 25 embryos. At 0.25 mg/mL, only fraction D had a significantly elevated mortality level of 11 dead/25 total embryos as compared to the solvent control level of 4/25.

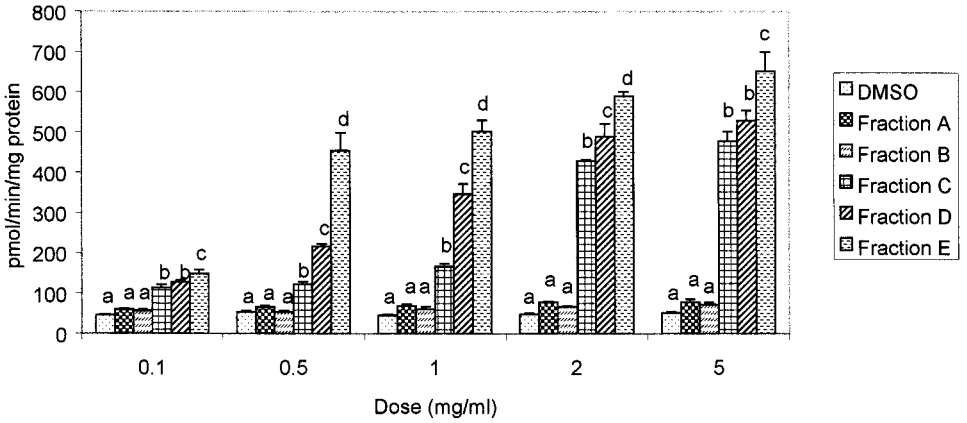

FIGURE 1. Activity of ethoxyresorufin-*O*-deethylease following induction by fractions A−E. Responses measured in rat liver Clone 9 cells after 24 h of treatment. Letters indicate similar responses at $p < 0.05$. Results compared using a one-way analysis of variance followed by Tukey's test.

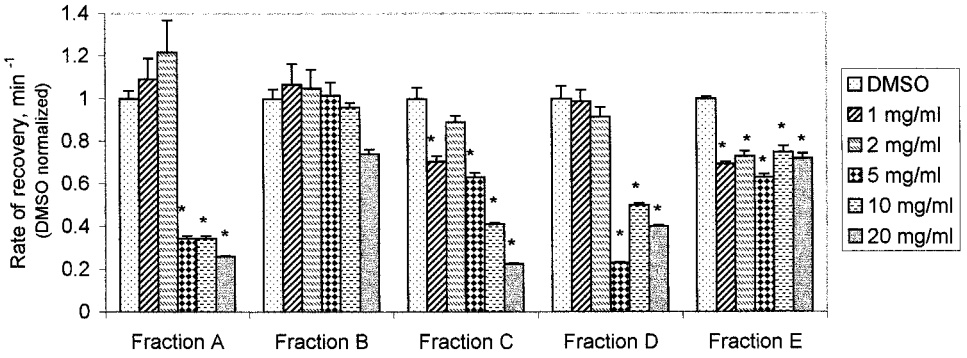

FIGURE 2. Inhibition of gap junction intercellular communication by fractions A−E. Responses measured in rater liver Clone 9 cells after 24 h of treatment. Asterisks indicate values that are significant from control at $p < 0.05$. Results compared using a one-way analysis of variance followed by Tukey's test.

TABLE 3. Number of Live Embryos and the Fraction of Embryos with Fat Streaked, Necrotic, or Green Livers as Measured by the Chicken Embryo Screening Test (CHEST)[a]

	live embryos/fraction damaged					
dose (mg/kg)	DMSO control	fraction A	fraction B	fraction C	fraction D	fraction E
0.125	21/0.05[a]	19/0.00[a]	22/0.05[a]	20/0.15[b]	22/0.32[b]	24/0.13[b]
0.25	21/0.00[a]	21/0.00[a]	23/0.13[a]	17/0.35[b]	14[b]/0.43[b]	20[b]/0.25[b]
0.5	22/0.05[a]	20/0.00[a]	22/0.09[a]	22/0.5[b]	22/0.45[b]	24/0.38[b]
1	23/0.00[a]	21/0.00[a]	24/0.00[a]	22/0.64[b]	23/0.39[b]	24/0.54[b]
2	17/0.00[a]	22/0.00[a]	19/0.00[a]	4[b]/0.86[b]	5[b]/0.81[b]	6[b]/0.78[b]

[a] Each group began with 25 embryos. Results are compared using the $R \times C \chi^2$ and Fisher exact tests. Superscript roman letters indicate responses that are significant between treatments at a given dose level at $p < 0.05$. [b] Mortality significantly different from control at $p < 0.05$.

Results of liver examinations from the CHEST assay are presented in Table 3. Livers that exhibited a green color or those exhibiting white necrotic patches or fatty streaks were defined as damaged. Liver damage in the fraction A and B groups was not different from solvent control. Fractions C−E produced significantly greater amounts of damage than the solvent control or fractions A and B. No significant differences were observed between fractions C, D, and E. At a dose of 0.5 mg/mL, for example, solvent control animals had one damaged liver per 22 living embryos. Fractions A and B had 0 damaged per 20 live embryos and 2 damaged per 22 live embryos, respectively. Fractions C−E had 11/22, 10/22, and 9/24, respectively.

No single method consistently predicted relative toxicity in the CHEST assay. At all doses, fractions A and B gave similar results but were significantly less toxic than fractions C−E. Total cPAHs seems to be the most accurate in that it predicted responses to fractions A and B that are similar to each other but significantly lower than those of fractions C−E. On an order of magnitude basis, total cPAHs place the fractions in the order C = D = E > A = B. PPs gave a similar prediction, although the value for fraction D was an order of magnitude greater than the values for fractions C and E.

GJIC. The results from measurement of intercellular communication in Clone 9 hepatic cells are presented in Figure 2. These data have been normalized to DMSO controls to simplify comparisons. Fraction A produced strong inhibition at doses of 5, 10, and 20 mg/mL. For these doses, the normalized rate of recovery was 0.345 ± 0.013, 0.345 ± 0.021, and 0.261 ± 0.003 min^{-1}, respectively. Fraction B produced no responses that were significantly different from DMSO control at any dose. Fraction C was significantly different from control at 1 mg/mL but not at 2 mg/mL. Doses of 5, 10, and 20 mg/mL of fraction C were all significantly different from control at $p < 0.05$. Fraction D produced responses that were significant at doses of 5, 10, and 20 mg/mL only with responses of 0.231 ± 0.004, 0.499 ± 0.011, and 0.403 ± 0.004, respectively. Fraction E induced responses that were significantly different from control at all doses tested. Doses of 1, 2, 5, 10, and 20 mg/mL of fraction E had responses of 0.693 ± 0.009, 0.728 ± 0.024, 0.630 ± 0.016, 0.749 ± 0.029 and 0.718 ± 0.024, respectively.

In GJIC assays, the results were difficult to interpret because of the unexpected effect of fraction A at doses of 5, 10, and 20 mg/mL. This result is not well understood but may be caused by the presence of unidentified compounds in this fraction. As indicated in Table 1, 82.3% of this fraction

was not accounted for by chemical analysis. It is possible that some of these unidentified compounds are exclusive to this fraction and had a marked effect on gap junction communication. Previous studies have indicated that anthracene and other low molecular weight hydrocarbons may have an appreciable effect on GJIC (*31*−*36*). Overall, fraction E seemed to be the most active in this assay because of the consistency of the observed effect at all doses. It does not, however, produce the strongest response as compared to responses produced by other fractions. Fractions A, C, and D gave the strongest inhibition of intercellular communication.

TEFs, PPs, total cPAHs, and total measured PAHs all failed to consistently predict the results obtained in this assay. More than 70% of each of the PAH fractions was unidentified based on GC/MS analysis. Other compounds present in the fractions may be producing effects on cell-to-cell communication and thereby complicating the interpretation of results. Further work with mixtures composed only of known PAHs could help to explain these results.

None of the PAH fractions consistently produced the maximum level of toxicity as measured across a variety of end points. PAH interactions that can influence the carcinogenic process may range from a simple affect on cellular uptake to compound interactions affecting metabolism or binding with critical proteins in the cell. In addition, as an individual ages, spontaneous mutations may play a role in initiating cells thereby making PAHs that act primarily by promotion effective as carcinogens. This also implies that, at the promotional stages of cancer, PAHs do not need to be metabolized to be biologically active. Under these circumstances, EROD activity may not accurately account for such toxicities. Attempts to define the toxicities of PAHs relative to BaP are also complicated by the fact that some cPAHs are capable both initiating and promoting tumors. The U.S. EPA's IRIS database lists benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, chrysene, and dibenz[*a*,*h*]anthracene as being able to promote tumors they initiate (*37*−*41*). This ability to act via distinct pathways to produce a single end point of cancer results in a large amount of uncertainty when attempting to predict responses of biological systems. Success with TEFs for compounds based on dioxin-like activity is the result of having a single, well-defined end point of Ah receptor affinity. For risk assessments of PAH carcinogenesis, the problem is perhaps too complex for a simple ranking system to completely account for all behaviors. PAH risk assessment would be improved by mechanistic studies providing a better understanding of complex mixture interactions.

The differential toxicity observed in various biological tests used in this study, as compared to chemical analysis, may also be the result of ignoring certain classes of toxic compounds while including other compounds of low toxicity. Alkylated chrysenes, for example, are known to have potential carcinogenic activities, yet they are not included in any TEF-like system. While PAHs such as these are excluded, compounds such as acenaphthene, acenaphthylene, anthracene, and phenanthrene, which have rarely been found to exhibit carcinogenic activity, are included. Further investigations into the activity of poorly characterized PAHs that are suspected to have some activity, such as the alkylated chrysenes, could greatly improve the predictive value of TEFs and PPs.

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